# EXHIBITS

Exhibit A

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 99.6 | 92.4 | 92.2 | 92.4 | 98.8 | 98.6 | 98.4 | 93.6 | 96.8 | 97.2 | 98.4 | 94.0 | 99.6 | 1 |
| 2 | | | 92.6 | 92.4 | 92.6 | 99.2 | 98.2 | 98.4 | 93.8 | 96.6 | 97.2 | 98.0 | 93.8 | 99.2 | 2 |
| 3 | | | | 99.8 | 99.8 | 93.0 | 93.4 | 93.4 | 98.8 | 95.6 | 95.0 | 91.0 | 96.2 | 92.0 | 3 |
| 4 | | | | | 99.6 | 92.8 | 93.2 | 93.2 | 98.6 | 95.4 | 94.8 | 90.8 | 96.0 | 91.8 | 4 |
| 5 | | | | | | 93.0 | 93.4 | 93.4 | 98.8 | 95.6 | 95.0 | 91.0 | 96.2 | 92.0 | 5 |
| 6 | | | | | | | 97.4 | 98.0 | 94.2 | 97.0 | 97.6 | 97.2 | 94.2 | 98.4 | 6 |
| 7 | | | | | | | | 99.0 | 94.2 | 95.4 | 95.8 | 97.4 | 94.2 | 98.2 | 7 |
| 8 | | | | | | | | | 94.2 | 95.8 | 96.0 | 96.8 | 94.2 | 98.0 | 8 |
| 9 | | | | | | | | | | 96.8 | 96.2 | 92.2 | 97.0 | 93.2 | 9 |
| 10 | | | | | | | | | | | 97.0 | 95.2 | 96.8 | 96.4 | 10 |
| 11 | | | | | | | | | | | | 95.8 | 96.4 | 96.8 | 11 |
| 12 | | | | | | | | | | | | | 92.8 | 98.0 | 12 |
| 13 | | | | | | | | | | | | | | 94.4 | 13 |
| 14 | | | | | | | | | | | | | | | 14 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |

1. Phl p 4 SEQ ID 2.pro
2. Phl p 4 SEQ ID 4.pro
3. Phl p 4 SEQ ID 6.pro
4. Phl p 4 patent clone 1.pro
5. Phl p 4 patent clone 2.pro
6. Phl p 4 patent clone 3.pro
7. Phl p 4 patent clone 4.pro
8. Phl p 4 patent clone 5.pro
9. Phl p 4 patent clone 6.pro
10. Phl p 4 patent clone 7.pro
11. Phl p 4 patent clone 8.pro
12. Phl p 4 patent clone 9.pro
13. Phl p 4 patent clone 10.pro
14. Phl p 4 patent clone 11.pro

**Alignment of the Phl p 4 sequences**

The homology of the sequences (% identical amino acids) was between 90.8 and 99,8 %.
The homology of the sequences of SEQ ID 2 and SEQ ID 6 was 92.4 %.

Exhibit B

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 99.3 | 85.4 | 84.7 | 85.4 | 96.5 | 99.3 | 98.6 | 85.4 | 89.6 | 90.3 | 94.4 | 84.7 | 100.0 | 1 |
| 2 | | | 85.4 | 84.7 | 85.4 | 97.2 | 98.6 | 99.3 | 85.4 | 89.6 | 91.0 | 93.8 | 84.7 | 99.3 | 2 |
| 3 | | | | 99.3 | 100.0 | 86.8 | 84.7 | 84.7 | 100.0 | 95.8 | 94.4 | 80.6 | 97.2 | 85.4 | 3 |
| 4 | | | | | 99.3 | 86.1 | 84.0 | 84.0 | 99.3 | 95.1 | 93.8 | 79.9 | 96.5 | 84.7 | 4 |
| 5 | | | | | | 86.8 | 84.7 | 84.7 | 100.0 | 95.8 | 94.4 | 80.6 | 97.2 | 85.4 | 5 |
| 6 | | | | | | | 95.8 | 97.9 | 86.8 | 91.0 | 92.4 | 91.0 | 86.1 | 96.5 | 6 |
| 7 | | | | | | | | 97.9 | 84.7 | 88.9 | 89.6 | 95.1 | 84.0 | 99.3 | 7 |
| 8 | | | | | | | | | 84.7 | 88.9 | 90.3 | 93.1 | 84.0 | 98.6 | 8 |
| 9 | | | | | | | | | | 95.8 | 94.4 | 80.6 | 97.2 | 85.4 | 9 |
| 10 | | | | | | | | | | | 90.3 | 84.0 | 93.8 | 89.6 | 10 |
| 11 | | | | | | | | | | | | 85.4 | 93.1 | 90.3 | 11 |
| 12 | | | | | | | | | | | | | 80.6 | 94.4 | 12 |
| 13 | | | | | | | | | | | | | | 84.7 | 13 |
| 14 | | | | | | | | | | | | | | | 14 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |

1. Phl p 4 SEQ ID 2.pro
2. Phl p 4 SEQ ID 4.pro
3. Phl p 4 SEQ ID 6.pro
4. Phl p 4 patent clone 1.pro
5. Phl p 4 patent clone 2.pro
6. Phl p 4 patent clone 3.pro
7. Phl p 4 patent clone 4.pro
8. Phl p 4 patent clone 5.pro
9. Phl p 4 patent clone 6.pro
10. Phl p 4 patent clone 7.pro
11. Phl p 4 patent clone 8.pro
12. Phl p 4 patent clone 9.pro
13. Phl p 4 patent clone 10.pro
14. Phl p 4 patent clone 11.pro

**Alignment of a 144 amino acid fragment (amino acids 219-362) of the Phl p 4 sequences**

The homology of the sequences (% identical amino acids) was between 79.9 and 100 %.
The homology of the sequences of SEQ ID 2 and SEQ ID 6 was 85.4 %.

Exhibit C

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 100.0 | 78.8 | 75.8 | 78.8 | 100.0 | 100.0 | 100.0 | 78.8 | 97.0 | 78.8 | 90.9 | 72.7 | 100.0 | 1 |
| 2 | | | 78.8 | 75.8 | 78.8 | 100.0 | 100.0 | 100.0 | 78.8 | 97.0 | 78.8 | 90.9 | 72.7 | 100.0 | 2 |
| 3 | | | | 97.0 | 100.0 | 78.8 | 78.8 | 78.8 | 100.0 | 81.8 | 100.0 | 72.7 | 90.9 | 78.8 | 3 |
| 4 | | | | | 97.0 | 75.8 | 75.8 | 75.8 | 97.0 | 78.8 | 97.0 | 69.7 | 87.9 | 75.8 | 4 |
| 5 | | | | | | 78.8 | 78.8 | 78.8 | 100.0 | 81.8 | 100.0 | 72.7 | 90.9 | 78.8 | 5 |
| 6 | | | | | | | 100.0 | 100.0 | 78.8 | 97.0 | 78.8 | 90.9 | 72.7 | 100.0 | 6 |
| 7 | | | | | | | | 100.0 | 78.8 | 97.0 | 78.8 | 90.9 | 72.7 | 100.0 | 7 |
| 8 | | | | | | | | | 78.8 | 97.0 | 78.8 | 90.9 | 72.7 | 100.0 | 8 |
| 9 | | | | | | | | | | 81.8 | 100.0 | 72.7 | 90.9 | 78.8 | 9 |
| 10 | | | | | | | | | | | 81.8 | 87.9 | 75.8 | 97.0 | 10 |
| 11 | | | | | | | | | | | | 72.7 | 90.9 | 78.8 | 11 |
| 12 | | | | | | | | | | | | | 69.7 | 90.9 | 12 |
| 13 | | | | | | | | | | | | | | 72.7 | 13 |
| 14 | | | | | | | | | | | | | | | 14 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |

1 Phl p 4 SEQ ID 2.pro
2 Phl p 4 SEQ ID 4.pro
3 Phl p 4 SEQ ID 6.pro
4 Phl p 4 patent clone 1.pro
5 Phl p 4 patent clone 2.pro
6 Phl p 4 patent clone 3.pro
7 Phl p 4 patent clone 4.pro
8 Phl p 4 patent clone 5.pro
9 Phl p 4 patent clone 6.pro
10 Phl p 4 patent clone 7.pro
11 Phl p 4 patent clone 8.pro
12 Phl p 4 patent clone 9.pro
13 Phl p 4 patent clone 10.pro
14 Phl p 4 patent clone 11.pro

**Alignment of a 33 amino acid fragment (amino acids 219-251) of the Phl p 4 sequences**

The homology of the sequences (% identical amino acids) was between 69.7 and 100 %.
The homology of the sequences of SEQ ID 2 and SEQ ID 6 was 78.8 %.

# Characterization of Phl p 4, a major timothy grass *(Phleum pratense)* pollen allergen

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**Background:** *Group 4 grass pollen allergens represent glycoproteins with a molecular weight of 50 to 60 kd, which are present in many grass species. Almost 75% of patients allergic to grass pollen display IgE reactivity to group 4 allergens, which hence can be regarded as major grass pollen allergens.*

**Objective:** *In this study attempts were made to obtain information regarding the immunologic properties, localization, and occurrence of Phl p 4 and related allergens.*

**Methods:** *Phl p 4 was detected in timothy grass pollen extracts by immunoblotting with serum IgE and monoclonal antibodies and was localized in pollen by immunelectron microscopy. A peptide sequence from Phl p 4 was obtained by amino acid sequencing. The resistance of Phl p 4 against trypsin was analyzed after trypsin treatment of timothy grass pollen extracts with serum IgE and monoclonal antibodies. Cross-reactivities between Phl p 4 and Amb a 1, the major allergen of ragweed, were studied by using monoclonal antibodies and by IgE-inhibition studies.*

**Results:** *Phl p 4 was characterized as a trypsin-resistant major timothy grass pollen allergen. By immunelectron microscopy Phl p 4 was localized in the exine, cytoplasm, and amyloplast of timothy grass pollen. Significant sequence similarities of a Phl p 4 10 amino acid peptide with Amb a 1, the major ragweed allergen, could be found. The immunologic similarity of Phl p 4 and Amb a 1 was confirmed by cross-reactivity of monoclonal antibodies and patients' IgE.*

**Conclusion:** *Phl p 4 represents a trypsin-resistant major timothy grass pollen allergen with immunologic similarities to the major ragweed allergen Amb a 1 and therefore must be considered an important cross-reactive component in grass pollen and weed pollen allergy.*
*(J Allergy Clin Immunol 1996;98:189-98.)*

*Key words: Grass pollen allergy, Phl p 4, trypsin resistance, monoclonal antibodies, cross-reactivity, Amb a 1, ragweed allergy, pollen-specific expression*

Grass pollen allergy has a high prevalence in Europe, America, and Australia where up to 70% of patients with type I allergy display IgE reactivity to grass pollen allergens.[1] Group 1, group 2/3, and group 5 grass pollen allergens were identified as major allergens that are present in most grass species.[2-4] Almost 95% of patients allergic to grass pollen show IgE specific for group 1 allergens, 80% react with group 5 allergens, and 60% to 70% display IgE reactivity to group 2/3 grass pollen allergens.[5] Although these major grass pollen allergens are well described and the corresponding complementary DNAs have been isolated and expressed in *Escherichia coli* as functional allergens,[6-13] little is known about group 4 grass pollen allergens. Group 4 grass pollen allergens were described as glycoproteins with molecular weight

*Abbreviations used*

| | |
|---|---|
| mAb: | Monoclonal antibody |
| PBS: | Phosphate-buffered saline |
| SDS-PAGE: | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |

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Supported by grant S06703 of the Austrian Science Foundation.
Received for publication July 10, 1995; revised Oct. 17, 1995; accepted for publication Oct. 18, 1995.

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0091-6749/96 $5.00 + 0 1/1/70334

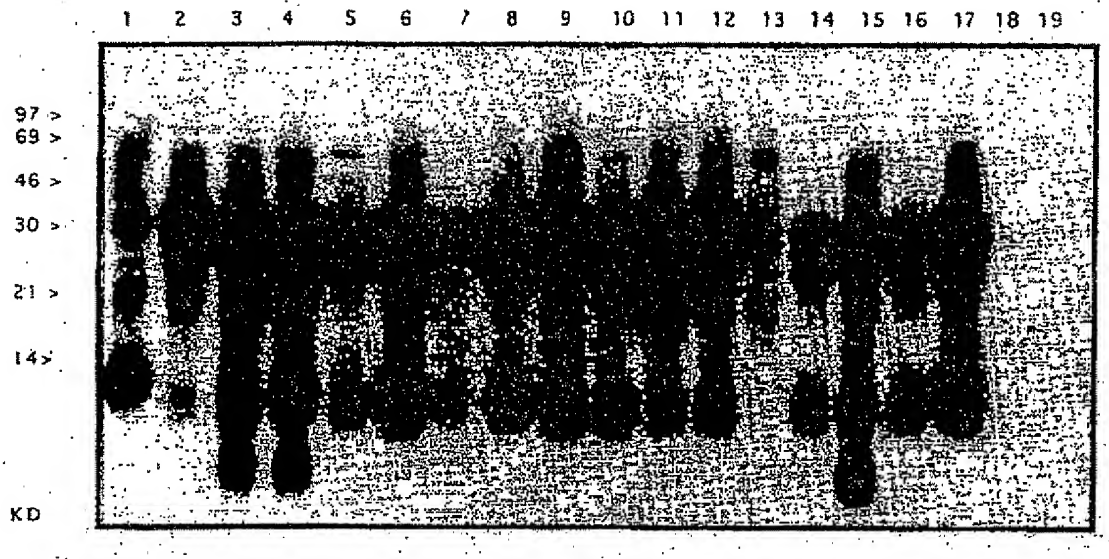


FIG. 1. IgE reactivity of sera from patients allergic to grass pollen to nitrocellulose-blotted timothy grass pollen extract. Sera from 17 patients allergic to grass pollen *(lanes 1 to 17)* were probed for IgE reactivity with nitrocellulose-blotted timothy grass pollen extract. The serum from a nonallergic individual was included as a control *(lane 18)*, and in *lane 19* buffer instead of serum was used (buffer control). Molecular weights are indicated at left.

of 50 to 60 kd, which are present in different grass species (e.g., ryegrass, Bermuda grass, timothy grass).[14-18] According to IgE-immunoblotting studies done with timothy grass pollen extract, up to 75% of patients allergic to grass pollen were found to display IgE reactivity to group 4 allergens.[5] Group 4 grass pollen allergens therefore belong to the major grass pollen allergens. On the other hand, IgE adsorption studies done with a combination of four recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5, and recombinant timothy grass profilin) have indicated that rather low levels of timothy grass pollen–specific IgE are directed against group 4 and other grass pollen allergens.[19]

To obtain information regarding group 4 allergens, immunoblot analyses with sera from patients allergic to grass pollen and monoclonal antibodies (mAbs) specific for Phl p 4[18] were done. In addition, the localization of Phl p 4 in timothy grass pollen was studied by immunelectron microscopy. Digestion of timothy grass pollen extracts with trypsin revealed a remarkable resistance of Phl p 4 against proteolysis. The amino acid sequence of a decapeptide derived from Phl p 4 showed significant homology with the major ragweed allergens, Amb a 1 and Amb a 2.[20, 21] Immunologic similarity of Phl p 4 and Amb a 1 was demonstrated by cross-reactivity of Phl p 4–specific mAbs and IgE inhibition studies.

## METHODS

### Allergen extracts and mAbs

Timothy grass *(Phleum pratense)* pollen and ragweed *(Ambrosia trifida)* pollen were purchased from Allergon (Välinge, Sweden). Two grams of pollen was extracted in 50 ml of distilled water for 2 hours at room temperature. The protein extracts were then centrifuged at 30,000 *g* for 30 minutes at 4° C, and supernatants were lyophilized and stored at −20° C until use.[22] The mAbs with specificity for group 4 allergens were described.[18]

### Characterization of patients allergic to grass pollen

Sera from allergic patients were characterized by RAST, skin testing, and case history regarding grass pollen (timothy grass) and weed pollen (mugwort, ragweed) allergy as previously described.[5] In addition, sera were tested for reactivity with recombinant timothy grass pollen allergens Phl p 1, Phl p 2, and Phl p 5 and birch profilin to determine each patient's allergogram.[5]

### Trypsin digestion of grass pollen extracts

Approximately 0.5 μg of timothy grass pollen extract was dissolved in 100 ml Tris-buffered saline (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.5) and digested with 10 μg of trypsin (Boehringer, Mannheim, Germany) at 37° C for 1 hour. The reaction was stopped by addition of 60 μl of sodium dodecylsulfate sample buffer[23] and boiling for 5 minutes.

Forty microliters of the mixture and of a sample that was prepared in the same way without addition of trypsin

were loaded onto a 9% sodium dodecylsulfate–polyacrylamide gel.[23] Gels were either stained with Coomassie Blue (Bio-Rad, Richmond, Calif.)[24] to visualize proteins or blotted onto nitrocellulose[25] for antibody detection.

## Determination of the amino acid sequence of a Phl p 4 peptide

Timothy grass pollen extract was separated by preparative sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 9% gel to obtain optimal separation of proteins in the 40 to 60 kd molecular weight range. Proteins were then transferred to Immobilon-polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.) by electroblotting.[25] Immobilon membranes were stained with Coomassie Blue according to the manufacturer's advice, and the position of group 4 allergens was marked. According to the amino acid analysis, 111 pmol of the protein was subjected to a digestion procedure. As controls, a piece of blank polyvinylidene difluoride membrane and transferrin were digested as well. The membranes were incubated in 100 µl of digest buffer (1% hydrogenated Triton X-100, 10% $CH_3CN$, 100 mmol/L Tris, pH 8.0) by adding lysyl endopeptidase (Boehringer) to a final concentration of 12 µg/ml for 24 hours at 37° C. After the digestion, the sample was separated on a Vydac C18 2.1 column (Millipore Corp.). Peak 90, which on the basis of the high-pressure liquid chromatography profile appeared homogenous, was sequenced by using a Beckman Analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The digestion, peptide purification, and sequencing were done as a commercial service at the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, Conn.).

## Purification of Phl p 4 by ion-exchange chromatography and preparative Western blotting

Phl p 4 was purified by ion-exchange chromatography as described by Fahlbusch et al.[26] and by preparative Western blotting. For the latter, timothy grass pollen extract (0.5 mg/cm gel) was separated by 9% preparative SDS-PAGE[23] and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany).[25]

## Immunelectron microscopic localization of Phl p 4 in timothy grass pollen

Timothy grass pollen grains were exposed to acrolein vapor for 7 days at room temperature, then dehydrated in dimethoxypropane and absolute ethanol for 4 hours each and embedded in Lowicryl K4M[27] with three intermediate steps of ethanol-Lowicryl (2:1, 1:1, 1:2) between. The temperature was lowered to −35° C, and polymerization was done by UV irradiation. Ultrathin sections were cut and then incubated on drops of the following solutions: (1) 5% bovine serum albumin in phosphate-buffered saline (PBS) for 5 minutes, (2) mAb 3C4, diluted 1:160 in PBS, for 2 hours, and (3) PBS and Tris, three times for 5 minutes each. Incubation was done in a moist chamber at room temperature with PBS (pH 7.4) and Tris buffer consisting of 20 mmol/L Tris, 20 mmol/L $NaN_3$, 225 mmol/L NaCl, and 1% wt/vol bovine serum albumin (pH 8.2). In control experiments, the specific primary antibody was replaced by another mAb of irrelevant specificity. Moreover, in another control experiment, the gold-coupled secondary antibody was applied to the sections without prior incubation with the primary antibody as described by Grote et al.[27]

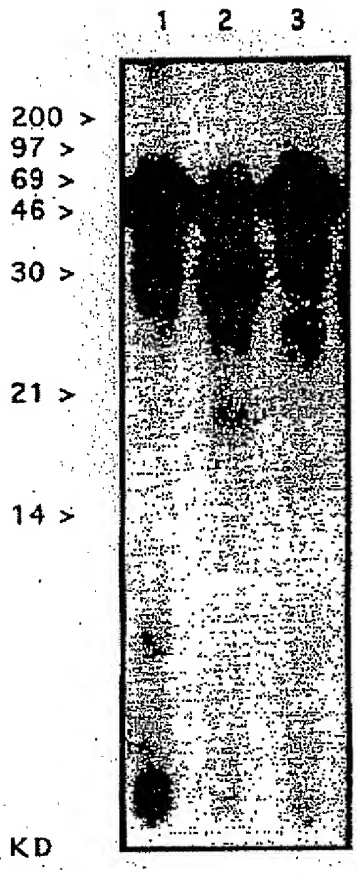


FIG. 2. Reactivity of Phl p 4–specific mAbs with nitrocellulose-blotted timothy grass pollen extract. Three murine mAbs specific for Phl p 4 (*lane 1*, 3C4; *lane 2*, 2D8; *lane 3*, 5H1) were probed with nitrocellulose-blotted timothy grass pollen extract.

## Immunoblotting with serum IgE and mAbs

Pollen extracts (0.5 mg/cm gel) were separated by preparative 12% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell). Strips of about 5 mm in width were cut from the membranes and incubated two times for 5 minutes and once for 30 minutes in buffer A (50 mmol/L Naphosphate: pH 7.5, 0.5% vol/vol Tween

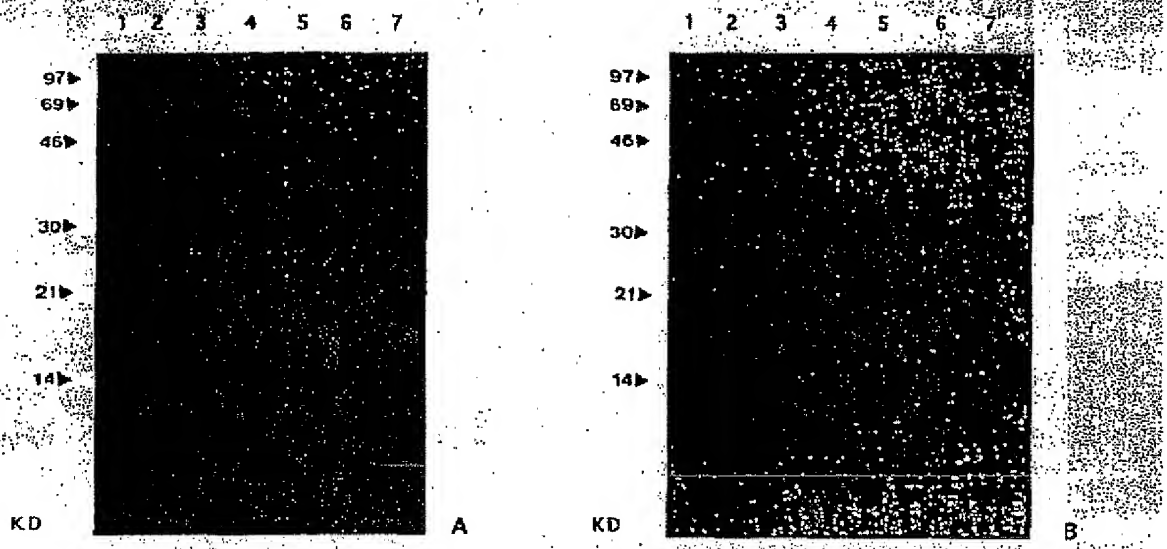


FIG. 3. Preadsorption of sera with a mixture of recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin reduces IgE binding to nitrocellulose-blotted timothy grass pollen extract. Sera from seven patients allergic to grass pollen *(lanes 1 to 7)* were preadsorbed with a mixture of recombinant allergens (A) or with a comparable amount of a control protein (dog albumin, negative control; B) and then incubated with nitrocellulose-blotted timothy grass pollen extract.

20, 0.5% wt/vol bovine serum albumin, 0.05% wt/vol $NaN_3$). Nitrocellulose strips were incubated with sera from allergic patients (diluted 1:10 in buffer A) or with hybridoma supernatants (diluted 1:5 in buffer A) overnight at 4° C. Strips were then washed as described previously. Bound IgE was detected with 1:10 diluted iodine 125–labeled anti-IgE antibodies (Pharmacia, Uppsala, Sweden). Bound murine mAbs were detected with a 1:500 diluted $^{125}I$-labeled sheep anti-mouse antiserum (Amersham, Buckinghamshire, U.K.) or with an alkaline phosphatase–coupled rabbit anti-mouse antiserum (Dianova, Hamburg, Germany). For the detection of bound radiolabeled antibodies, Kodak X-OMATS film and intensifying screens (Kodak, Heidelberg, Germany) were used.

### IgE absorption experiments

IgE inhibitions were done as described by Vrtala et al.[13] In brief, serum samples from seven patients allergic to timothy grass pollen were depleted of Phl p 1, Phl p 2, Phl p 5, and profilin-specific IgE by preincubation with 10 μg of each recombinant allergen (recombinant birch profilin, which shares most IgE epitopes with timothy grass profilin, was used[28]) per milliliter of 1:10 diluted serum. Purified natural Phl p 4 was used for preadsorptions at a concentration of 5 μg/ml of 1:10 diluted serum. After preincubation, the serum dilutions were diluted 1:2 and incubated with the blotted extracts, and bound IgE was detected.

## RESULTS

### IgE reactivity of sera from patients allergic to grass pollen

Fig. 1 shows the IgE binding of sera from 17 patients allergic to grass pollen to nitrocellulose-blotted timothy grass pollen extract. All patients' sera showed IgE reactivity to proteins of approximately 30 kd, which contain group 1 and group 5 allergens; 16 sera showed reactivity to proteins of 10 to 14 kd, which represent group 2/3 and timothy grass profilin; and three sera displayed reactivity to proteins of about 8 kd, which may represent calcium-binding allergens from timothy grass.[29] Fourteen sera bound to proteins of approximately 22 kd, which may represent group 6 allergens[30] or cleavage products of group 5 allergens. Fourteen sera reacted with proteins ranging from 46 to 90 kd, which were described as group 4 allergens.[31-33] According to the percentage of sera that reacted with 60 kd proteins in Fig. 1 (82%) and in previous studies done with 98 sera (75%),[5] group 4 allergens may be considered major grass pollen allergens. The typing with recombinant allergens (see Methods) showed that the serum from patient 1 contained no IgE specific for Phl p 1, Phl p 2, and Phl p 5. The band at 14 kd is due to a reactivity with timothy grass pollen profilin.


FIG. 4. Immunelectron microscopic localization of Phl p 4 in timothy grass pollen. Ultrathin sections of timothy grass pollen grains after postembedding labeling with primary antibodies and gold-conjugated secondary antibodies. **A and B,** Sections were incubated with monoclonal anti-Phl p 4 antibody 3C4. **A,** Antibody binding sites were found within the exine part of the pollen wall (slightly tangentially cut) and in the cytoplasmic matrix. **B,** Phl p 4 is localized between the P-particles and within the amyloplasts. **C and D,** An antibody with irrelevant specificity was used as a control. The cell wall (cross section) and the cytoplasm showed a few nonspecifically adsorbed gold particles. *Bar* represents 0.2 μm. *A,* Amyloplast; *E,* exine; *I,* intine; *M,* mitochondrion; *P,* polysaccharide particle. (Original magnification ×50,000.)

| | | |
|---|---|---|
| Phl p 4 | I V A L P X G M L K | |
| Amb a 1.3 | V K V - - G - - I - | aa 173–182 |
| Amb a 1.2 | - K V C - G - - I - | |
| Amb a 1.1 | V K V N - G - L I - | |
| Amb a 2 | V K V - - G - R I - | |
| Zm58.2 | S K - H S G - - I R | |
| lat59 | - - E G N G - - V R | |
| lat56 | Y C T Y R S - L - R | |
| G10 | - - P T - G - L - R | |

FIG. 5. Sequence comparison of a decapeptide sequence of Phl p 4 with homologous peptides from the major ragweed allergen Amb a 1 and pollen-specific proteins. A decapeptide sequence obtained from Phl p 4 was compared with known protein sequences from the major ragweed allergens Amb a 1 and Amb a 2, a pollen-specific protein from *Zea mays* (Zm58), anther-expressed proteins from tomato (lat59, lat56), and a pollen-specific tobacco protein (G10). Identical amino acid residues are indicated by a *dash*. The amino acid in position X of the Phl p 4 peptide could not be determined.

## Murine mAbs specific for Phl p 4 detect proteins of different molecular weight in timothy grass pollen extract

Three different murine mAbs specific for Phl p 4 (3C4, 2D8, 5H1) were tested for reactivity to nitrocellulose-blotted timothy grass pollen extract, as shown in Fig. 2. All three mAbs bound strongly to a protein of about 55 to 60 kd. Additional binding to proteins of 69 kd and between 30 and 40 kd was observed. The mAbs in lanes 2 and 3 demonstrated bands at 19 to 20 kd and 24 to 25 kd. The binding pattern that was observed for the Phl p 4–specific mAbs was very similar to the IgE binding pattern observed with serum 1 in Fig. 1, which did not contain IgE specific for Phl p 1, Phl p 2, and Phl p 5.

## Depletion of Phl p 1, Phl p 2, Phl p 5, and profilin-specific IgE leads to a strong reduction of IgE binding to nitrocellulose-blotted timothy grass pollen extract

Fig. 3 shows the IgE binding of seven sera to nitrocellulose-blotted timothy grass pollen extract after adsorption with a combination of recombinant Phl p 1, Phl p 2, Phl p 5, and birch profilin (Fig. 3, *A*) and without preadsorption (Fig. 3, *B*). The weak IgE binding to group 4 allergens at 55 to 60 kd (lanes 3, 4, 6, and 7) was not significantly affected by the preadsorption, whereas strong reduction of the IgE binding to group 1 and 5 allergens at 30 kd was observed. At 1:20 serum dilutions, the binding to group 4 allergens was already very weak compared with the strong binding to group 1 and group 5 allergens, indicating that low levels of timothy grass pollen–specific IgE are directed against Phl p 4 and allergens other than those of groups 1 and 5.

## Localization of Phl p 4 in timothy grass pollen by immunoelectron microscopy

After incubation of timothy grass pollen sections with the mAb 3C4, Phl p 4 was found in the pollen wall and in the interior of the grain (Fig. 4, *A* and *B*). In the pollen wall, it was predominantly the exine part with its bacular cavities that was decorated with gold particles (Fig. 4, *A*). In the cytoplasmic interior of the grain, antibodies to group 4 allergen bound to the amyloplasts and the cytoplasmic matrix, as previously demonstrated for group 5 allergens.[11, 27] There was no specific labeling of the numerous polysaccharide inclusions (P-particles) or cell organelles, such as mitochondria (Fig. 4, *B*). Control experiments replacing the group 4–specific antibody with an antibody of irrelevant specificity showed a low degree of background staining in all pollen compartments (Fig. 4, *C* and *D*). Incubation of the sections with the gold-coupled secondary antibody alone likewise yielded a small number of nonspecifically bound gold particles (data not shown).

## A peptide derived from Phl p 4 showed significant sequence homology with the Amb a 1/2 major ragweed allergen family

A decapeptide sequence of Phl p 4, which was obtained by amino acid sequencing, could be compared with the National Center for Biological Information's databases (i.e., Protein Identification Resource, Genpept, and Swiss Protein databases). Fig. 5 shows significant sequence similarities of the Phl p 4 peptide with peptides from the major allergen family of ragweed (Amb a 1, Amb a 2).[20, 21] Additional homologies were found with pollen-specific, expressed proteins from *Zea mays* (Zm58),[31] tomato (lat59, lat56),[32] and tobacco pollen (G10).[33] The peptides were located at comparable portions in the mature proteins, and all homologous proteins had a comparable molecular weight and were described to be homologous to pectate lyases from the plant pathogen *Erwinia*.[34] All Amb a 1 homologous proteins were reported to be highly expressed in pollen tissue and occurred in monocot plants (maize), as well as in dicot plants (ragweed, tomato, and tobacco).

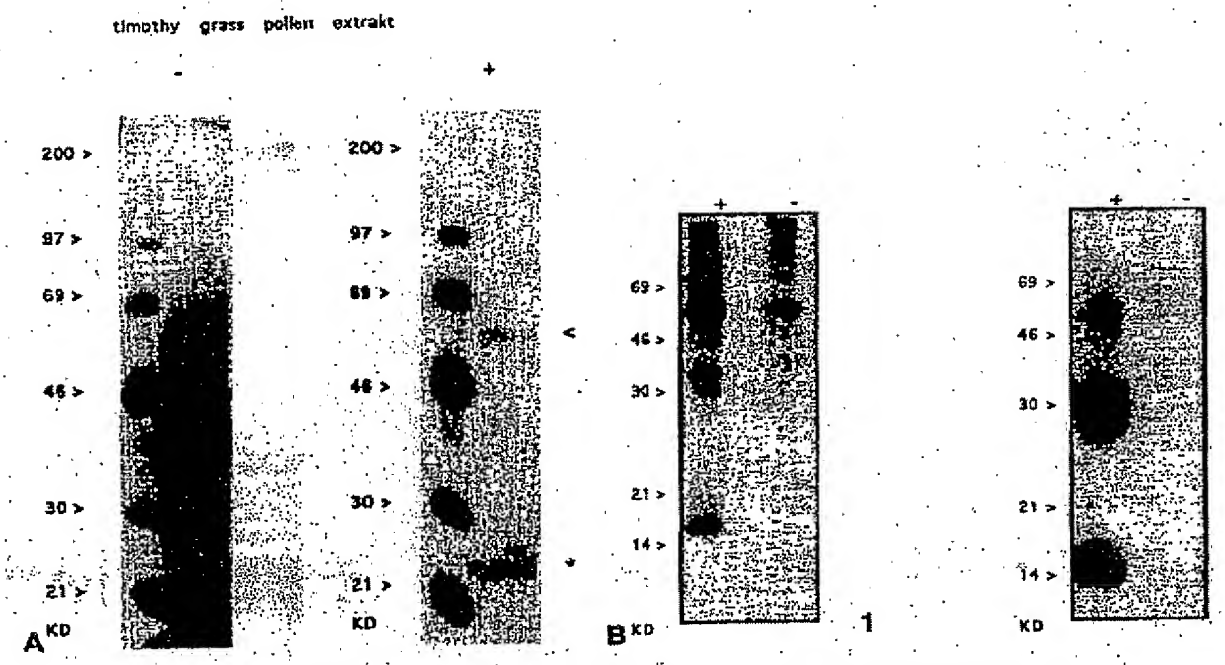


FIG. 6. A, Coomassie Blue–stained SDS-PAGE containing trypsin-digested and untreated timothy grass pollen extract. Timothy grass pollen extract was digested with trypsin (+) or untreated (−) and separated by 9% SDS-PAGE. The gel was then stained with Coomassie Blue to visualize proteins. *Arrow* indicates the position of a 55 to 60 kd trypsin-resistant protein, and *asterisk* indicates trypsin. B, IgE reactivity of sera from patients allergic to grass pollen with trypsin-digested and untreated timothy grass pollen extract. Sera from two patients (1 and 2) were used to detect nitrocellulose-blotted timothy grass pollen extract, which was digested with trypsin (+) or which had been untreated (−).

## Phl p 4 represents a trypsin-resistant allergen

Difficulties in obtaining sufficient amounts of tryptic peptides of Phl p 4 for amino acid sequencing indicated trypsin resistance of Phl p 4, as observed for Art v 1.[35] To determine whether Phl p 4 might represent a trypsin-resistant allergen, timothy grass pollen extract was digested with trypsin and probed with sera from patients allergic to grass pollen. The Coomassie Blue–stained SDS-PAGE in Fig. 6, *A*, shows that after cleavage of timothy grass pollen extract with trypsin, only one prominent band of about 55 to 60 kd remained undigested. The additional band at 25 kd represented trypsin (indicated with an asterisk in Fig. 6, *A*). Fig. 6, *B*, shows the IgE reactivity of sera from two patients with trypsinized and untreated timothy grass pollen extract. Patient 1 had IgE antibodies specific for Phl p 4 and profilin. Trypsin digestion of timothy grass pollen extract abolished IgE binding to profilin at 14 kd, whereas IgE binding to Phl p 4 at 55 to 60 kd was still observed. In the case of patient 2 who displayed IgE reactivity against Phl p 1, Phl p 2, Phl p 4, and Phl p 5, the IgE binding to group 1, 2, and 5 allergens was abolished after trypsinization of timothy grass pollen extract, whereas binding to Phl p 4 was still detectable. Similar experiments were done with mAbs specific for Phl p 1, Phl p 5, and Phl p 4; the outcome indicated that in contrast to the other allergens, Phl p 4 represented a trypsin-resistant allergen (data not shown).

## Phl p 4 is immunologically related to the Amb a 1/2 family of major ragweed allergens

Possible immunologic similarities of Phl p 4 and Amb a 1 were investigated with mAbs and IgE-adsorption studies. In a first experiment it was demonstrated that two of three Phl p 4–specific murine mAbs cross-reacted with proteins of a comparable molecular weight in ragweed pollen extract (Fig. 7). Because proteins with significant sequence homology to Amb a 1 were also described to be present in maize pollen, cross-reac-

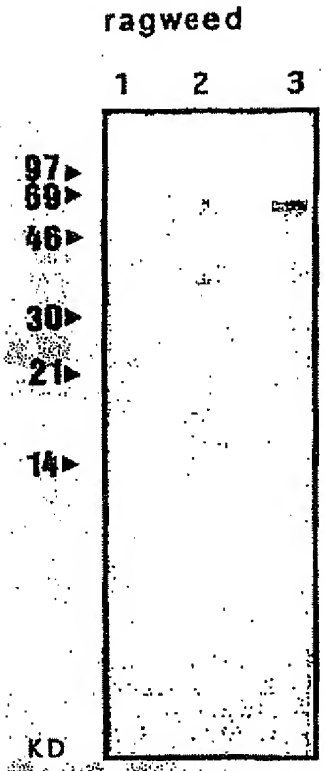


FIG. 7. Cross-reactivity of Phl p 4–specific mAbs with ragweed pollen extract. Three murine mAbs specific for Phl p 4 (*lane 1*, 2D8; *lane 2*, 3C4; *lane 3*, 5H1) were probed with nitrocellulose-blotted ragweed pollen extract.

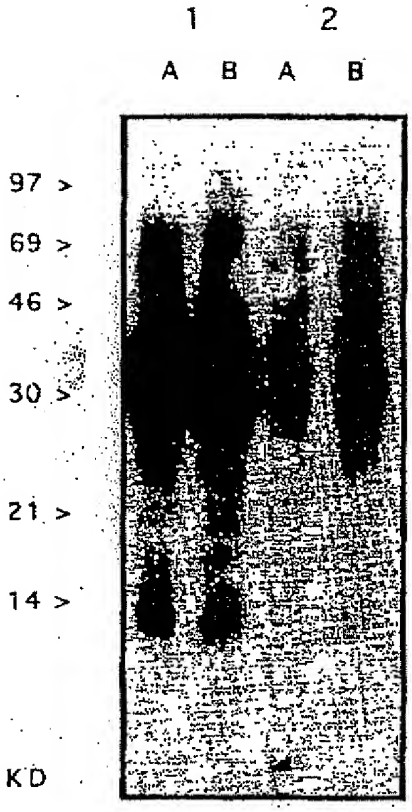


FIG. 8. Inhibition of patients' IgE binding to nitrocellulose-blotted ragweed pollen extract with purified Phl p 4. Sera from two patients allergic to grass pollen (1 and 2) were preadsorbed with purified Phl p 4 *(lane A)* or dog albumin *(lane B)* and then incubated with nitrocellulose-blotted ragweed pollen extract.

tivity of the Phl p 4–specific mAbs was tested with nitrocellulose-blotted maize pollen extract; the outcome indicated that the mAbs 2D8 and 5H1 bound to maize proteins of corresponding molecular weight (data not shown). It is hence likely that Phl p 4, Amb a 1, and their homologous proteins in maize (Zm58) and perhaps tomato (lat59) may represent a family of cross-reactive plant allergens.

To further substantiate the immunologic relationship of Phl p 4 and Amb a 1, IgE inhibition experiments were done with sera from patients with grass and weed pollen allergy. The presence of common IgE epitopes was investigated by using Phl p 4, which had been purified by chromatography or by preparative Western blotting. Fig. 8 shows that preincubation of sera from two patients with purified Phl p 4 substantially reduced IgE binding to proteins of 55 to 60 kd (patient 1) and to proteins of about 30 kd (patient 2) in ragweed pollen, whereas preincubation with an unrelated allergen, dog albumin (negative control),[36] did not affect the IgE binding. A similar inhibition of IgE binding to ragweed pollen extract was observed when the same sera were preadsorbed with Phl p 4, which had been isolated by preparative Western blotting (data not shown).

## DISCUSSION

Grass pollen allergens belong to the most potent elicitors of type I allergy in Europe, America, and Australia. The major grass pollen allergens—described as group 1, 2/3, and 5 allergens—have been well characterized by protein and immunochemical techniques and can be produced as functional recombinant allergens in *E. coli*. Much less is known about group 4 allergens, which occur as 50 to 60 kd proteins in different grass species and represent targets for IgE antibodies of up to 75% of patients allergic to grass pollen.[5] So far, studies have only described the presence of group 4 allergens in different grasses.[14-18]

When Phl p 4 was used as a representative allergen, the characterization of group 4 allergens was extended by proteinchemical, immunochemi-

cal, and immunohistologic techniques. This study reveals a remarkable resistance of Phl p 4 against trypsin digestion and an as yet unknown immunologic similarity of this allergen with the Amb a 1/2 major ragweed allergen family. The identification of Phl p 4 as a cross-reactive allergen in grasses and weeds might explain clinical symptoms of patients with grass pollen allergy on contact with weeds. In fact, all those patients whose sera reacted with group 4 allergens in the immunoblot shown in Fig. 1 had a positive case history, skin reaction, and serology test result indicative of weed pollen allergy.

On the one hand, IgE-immunoblotting experiments indicated that the 50 to 60 kd band, presumably representing Phl p 4, is recognized by the majority of sera from patients with grass pollen allergy. On the other hand, IgE-inhibition experiments showed that rather low amounts of grass pollen–specific IgE antibodies reacted with Phl p 4 and allergens other than Phl p 1,[8] Phl p 2,[9] Phl p 5,[13] or profilin.[28]

The rather low capacity of Phl p 4 to mount IgE responses is not due to the presence of low amounts of this allergen in grass pollen. Coomassie Blue staining of timothy grass pollen extracts identified Phl p 4 as an abundant protein. When immunelectron microscopy was used, dense labeling of Phl p 4 was found in the pollen grain sections. The allergen was localized in the cytoplasm as previously demonstrated for group 1 allergens, but the majority of the allergen was detected in the amyloplast as demonstrated for group 5 allergens.[27] Additional staining was observed in the inner part of the exine.

A significant sequence homology of a Phl p 4 decapeptide sequence and the major allergen family of ragweed, Amb a 1/2,[20, 21] was found. Homologies spanning the complete sequences of the Amb a 1/2 allergen family have already been described for a number of pollen-specific proteins from tobacco,[33] tomato,[32] and maize.[31] The peptides displaying sequence similarities with the Phl p 4 peptide were located at corresponding regions in the proteins, and all homologous proteins had a comparable molecular weight of 50 to 60 kd, which corresponds to the molecular weight observed for Phl p 4 in SDS-PAGE. Recently, the complete cDNA and deduced amino acid sequence of Cry j 1, the major allergen from Japanese cedar pollen, was shown to be highly homologous to the Amb a 1/2 ragweed allergen family.[37]

Despite these numerous reports describing sequence similarities of the Amb a 1/2 major allergen family with pollen-specific proteins and the major allergen of Japanese cedar pollen, Cry j 1, it is not known whether these sequence similarities may be responsible for immunologic cross-reactivities. Using Phl p 4–specific mAbs and IgE-inhibition experiments, we attempted to determine whether Phl p 4 might be immunologically related to the Amb a 1/2 allergens. Cross-reactivity of the mAbs with ragweed pollen extract, as well as IgE inhibition studies with Phl p 4, proved the presence of common epitopes.

Experiments showing that Phl p 4 represented a trypsin-resistant protein are of interest because they indicate that protease-resistant IgE epitopes are not exclusively due to carbohydrate moieties but can also be formed by the protein backbone of an allergen. This is of relevance in view of previous studies describing protease-resistant cross-reactive components in pollen and vegetable foods.[38] It is therefore not unlikely that Phl p 4 and Amb a 1/2 homologous allergens constitute a family of cross-reactive plant allergens as described previously for profilins.[39-42] The presence of common IgE epitopes of Phl p 4 with ragweed allergens may provide an explanation for the clinical observation that patients allergic to grass pollen frequently also have allergy to weed pollen.

We thank Dr. Joseph P. Mascarenhas for valuable discussions.

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*Clinical and Experimental Allergy*, 2000, Volume 30, pages 1395–1402

# The high molecular mass allergen fraction of timothy grass pollen (*Phleum pratense*) between 50–60 kDa is comprised of two major allergens: Phl p 4 and Phl p 13

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**Summary**

*Background* More than 70% of the patients allergic to grass pollen exhibit IgE-reactivity against the high molecular mass fraction between 50 and 60 kDa of timothy grass pollen extracts. One allergen from this fraction is Phl p 4 that has been described as a basic glycoprotein. A new 55/60 kDa allergen, Phl p 13, has recently been purified and characterized at the cDNA level.

*Objective* The relative importance of the two high molecular mass allergens has been characterized with respect to their IgE-binding frequency and capacity.

*Methods* Both high molecular mass allergens were biochemically purified and subjected to nitrocellulose strips. About 306 sera obtained from subjects allergic to grass pollens were used to determine specific IgE-binding frequency to Phl p 4 and Phl p 13. IgE-binding of allergens was quantified by ELISA measurements. Pre-adsorption of sera with purified allergens and subsequent incubation of nitrocellulose-blotted timothy grass pollen extract was performed to determine whether or not Phl p 4 and Phl p 13 represent the whole high molecular mass allergen fraction. Proteolytic stability of both allergens was investigated by addition of protease Glu-C.

*Results* More than 50% of 300 patients displayed IgE-binding with both allergens. Clear differences concerning the immunological properties of Phl p 4 and Phl p 13 were confirmed by individual IgE reactivities. Quantification of specific IgE for both allergens revealed comparable values. For complete inhibiton of IgE-binding in the high molecular mass range preincubation of sera with both allergens was necessary. Interestingly, inhibition of strong reacting sera with Phl p 13 eliminated not only reactivity of the 55/60 kDa double band, but in addition a 'background smear'. Whilst undenatured Phl p 4 was resistent to proteolytic digestion with Glu-C, native Phl p 13 was degraded rapidly.

*Conclusion* Phl p 4 and Phl p 13 are immunologically different and must both be considered as major allergens. They are judged to be important candidates for potential recombinant therapeutics that may provide a basis for improved immunotherapy.

*Keywords:* *Phleum pratense*, timothy grass pollen, Phl p 4, Phl p 13, major allergen

*Clinical and Experimental Allergy*, Vol. 30, pp. 1395–1402. Submitted 8 November 1999; revised 30 December 1999; accepted 25 January 2000.

## Introduction

Timothy grass pollen extracts are complex mixtures containing various allergenic components. Various allergens of *Phleum pratense* have been described in detail, including the major allergens Phl p 1 [1] and Phl p 5 [2]. These allergens exhibit molecular masses between 25 and 35 kDa. In addition, smaller timothy grass pollen allergens with molecular masses around 12 kDa have been investigated at the sequence level, for instance Phl p 2 [3] and Phl p 6 [4]. In contrast, little is known about the allergens with molecular sizes between 50 and 60 kDa, although 75% of grass allergic patients produce IgE specifically directed against these

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components [5]. One component designated as Phl p 4 has been described as a basic glycoprotein with a molecular mass of 55 kDa [6,7]. Monoclonal antibodies directed against this allergen have been generated [6], thus facilitating its detection and quantification [8]. Group 4 allergens were first described in *Lolium perenne* [9] and also characterized in other grass species, such as and *Dactylis glomerata* [10]. The high molecular mass allergen BG-60 from *Cynodon dactylon* shares at least size and isoelectric point with group 4 [11]. Leduc-Brodard *et al.* [10] have used mAbs to demonstrate cross-reactivity between group 4 allergens from various sources.

Recently, another timothy pollen allergen with a similar molecular size as Phl p 4 has been purified [12] and characterized at the cDNA level (EMBL accession no. AJ238848). Since this new allergen, designated Phl p 13, exibited different biochemical properties compared with Phl p 4, it was assumed that at least two different groups of allergens are present in the high molecular mass fraction of timothy grass pollen extract.

In order to estimate the importance of both high molecular allergens, they were purified, subjected to immunoblotting and analysed with 306 different sera derived from subjects allergic to grass pollens. Specific IgE was determined to evaluate of the binding capacities of each allergen. Investigations were also conducted to determine whether these two allergens alone were enable to effect complete inhibition of IgE-binding in the high molecular mass range.

## Materials and methods

### *Purification of timothy grass pollen allergens*

The purification of Phl p 1, group 2/3 and Phl p 13 was performed as described previously [12]. Briefly, a timothy grass pollen extract was separated by hydrophobic interaction chromatography (HIC) using phenyl sepharose HP (Pharmacia Biotech, Freiburg, Germany). The flow-through fraction was subsequentely concentrated with ultrafiltration devices Centriprep-10 (Millipore, Eschborn, Germany). Proteins of the flow-through fraction were finally purified using gel filtration with Superdex 75 (Pharmacia Biotech).

Phl p 4 was purified according to the procedures of Haavik *et al.* [6] and Fischer *et al.* [7] with some modifications. A timothy grass pollen extract obtained from 5 g pollens was subjected to G-25 gel filtration in phosphate buffer (20 mM, pH 7.0). The first peak was collected and applied to a 16/10 cation exchange column packed with Source 30S (Pharmacia Biotech) equilibrated with phosphate buffer. Elution of bound proteins was achieved using running buffer supplemented with 1 M NaCl. In order to remove trace amounts of Phl p 13, eluted proteins were subsequently adjusted to 20 mM Tris–HCl pH 8.0, 1 M $(NH_4)_2SO_4$ and subjected to hydrophic interaction chromatography. After elution of Phl p 4 with distilled water, final purification was performed using size exclusion chromatography with Superdex 75.

### *SDS-PAGE and immunoblotting*

Proteins were separated by SDS-PAGE (120 × 80 × 0.8 mm) with the buffer system of Laemmli [13]. After electrophoresis, proteins were transferred onto a supported nitrocellulose membrane (Sartorius, Göttingen, Germany) by semidry blotting for 30 min at 2 mA/cm$^2$. Immobilized proteins were either stained using india ink (Pelikan, Hannover, Germany) and afterwards AuroDye (Janssen Biochimica, Beerse, Belgium) [14] or incubated with mAbs or serum.

In order to screen multiple purified allergens with sera derived from allergic subjects, a Miniblot MN45 apparatus (Biometra, Göttingen, Germany) was used to apply allergens onto nitrocellulose membranes. Purified allergens (0.1 μg/μL), which were dissolved in 20 mM NaOH, were applied to the conduits of the apparatus and incubated for 30 min. After washing the conduits with distilled water, the membranes were blocked using Tris-buffered saline with 0.05% (v/v) Tween 20. For immunodetection, strips from the membranes (allergen strips) were incubated with sera or mAbs overnight.

### *Immunodetection and inhibition experiments*

Prior to immunodetection, membranes were blocked by treatment for 30 min with Tris-buffered saline (TBS, pH 7.4) supplemented with 0.05% Tween 20. Incubation with 1 : 20 diluted sera derived form allergic subjects or mAbs was carried out overnight. Identification of Phl p 4 was performed using monoclonal antibodies 5H1 and 3C4 [7], of group 1 allergens by mab 9C12 [15] and group 5 allergens using mab 1D11C8 [16,17]. After washing, bound antibodies were detected with alkaline phosphatase conjugated monoclonal mouse antihuman IgE (Allergopharma, Reinbek, Germany) or goat antimouse IgG/M with alkaline phosphatase (Dianova, Hamburg, Germany). The binding patterns were visualized by a substrate solution consisting of NBT/BCIP (Life Technologies) in 100 mM Tris–HCl-buffered saline solution (pH 9.5).

Inhibition experiments were conducted by adding 75 ng allergen per μL undiluted serum. After incubation for 30 min under gentle agitation, sera were diluted 1 : 20 in TBST and subsequently used for probing of nitrocellulose blotstrips carrying timothy grass pollen extract separated in a 12,5% SDS-PAGE.

*Determination of allergen-specific IgE*

Allergen-specific IgE levels were determined by EAST measurements with a specific 'IgE ELISA RV' kit (Allergopharma, Reinbek, Germany) containing alkaline phosphatase conjugated monoclonal mouse antihuman IgE antibodies. Experimental allergen discs carrying purified allergens were prepared according to the method of Ceska *et al.* [18]. In order to determine a value of kU/L reference sera and discs, which were included in the kit, were used to generate a standard curve. Results are displayed as means of duplicate determinations.

*Proteolytic digestion of purified allergens*

Lyophilized proteins were reconstituted in 25 mM $Na_2HPO4$, pH 7.3 at a concentration of 0.5 mg/mL. Proteolysis was performed by the addition of 0.25 μg Glu-C sequencing grade (Promega, Heidelberg, Germany) to 5 μg allergen and subsequent incubation at 37 °C for 2 h. The reactions were stopped by addition of reducing sodium dodecylsulphate sample buffer and boiling for 5 min

## Results

In order to investigate the relevance of the high moleculer weight allergen group between 50 000 and 60 000 of timothy grass pollens, the two known members Phl p 4 and Phl p 13 were biochemically purified. Phl p 4 was purified on the basis of its basic pI by ion exchange chromatography, whilst Phl p 13 was obtained after hydrophobic interaction chromatography in the flow-through fraction. In addition, Phl p 1 and group Phl p 2/3 were purified for control purposes. SDS-PAGE separation demonstrated that the allergens were purified to near homogeneity (Fig. 1).

After applying a timothy grass pollen extract and purified allergens onto nitrocellulose membranes, strips were incubated with sera derived from patients allergic to grass pollens and developed with mouse anti human-IgE mAb conjugated with alkaline phosphatase (Fig. 2). For the application of allergens different solutions were investigated, such as Tris-buffered saline and 20 mM NaOH. In both cases identical results were obtained regarding IgE-reaction patterns, but it was found that most efficient binding of allergens and consequently enhanced immunostaining occurred by using NaOH (data not shown).

Human serum albumin (HSA) was used as a negative control. Interestingly, the patients' sera revealed highly differentiated reaction patterns. In each case IgE-binding was observed with the timothy grass pollen extract, whereas reaction with purified allergens varied in nearly every possible combination. In the cases of the high molecular

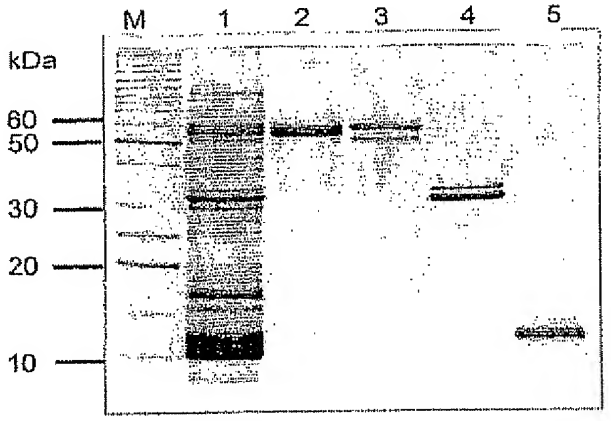


**Fig. 1.** Coomassie-stained SDS-PAGE of purified allergens showing separated molecular weight marker (M), timothy grass pollen extract (1), Phl p 4 (2), Phl p 13 (3), Phl p 1 (4) and group Phl p 2/3 (5).

mass allergens Phl p 4 and Phl p 13, IgE-binding was detected either for none, one or for both proteins, showing clear differences at the polyclonal IgE-level. The allergens are clearly immunologically distinct. Table 1 contains the IgE-reactivity of 306 sera investigated by allergen strips. Since the allergens exhibited more than 50% IgE-binding, they all meet the requirements for classification as major allergens, though the value for the high molecular mass allergens Phl p 4 and Phl p 13 was found to be only slightly above this arbitrary level. As expected, Phl p 1 displayed the highest IgE-binding frequency of about 95% [19]. The frequency obtained for group 2/3 also correlates with those reported in literature [20,21].

Allergen-specific IgE from eight sera was determined and a sufficient correlation between immunostaining of allergen strips and ELISA quantification was found throughout (Fig. 3).

Serum IgE for Phl p 4 was determined in the range of 0.3–15.5 kU/l, whereas specific IgE for Phl p 13 varied

**Table 1.** IgE-binding frequency of purified timothy grass pollen allergens

| Allergen | IgE-reactive sera ($n = 306$) | % of sera tested |
|---|---|---|
| Phl p 4 | 170 | 55.6 |
| Phl p 13 | 154 | 50.3 |
| Phl p 1 | 293 | 95.8 |
| Phl p 2/3 | 183 | 59.8 |

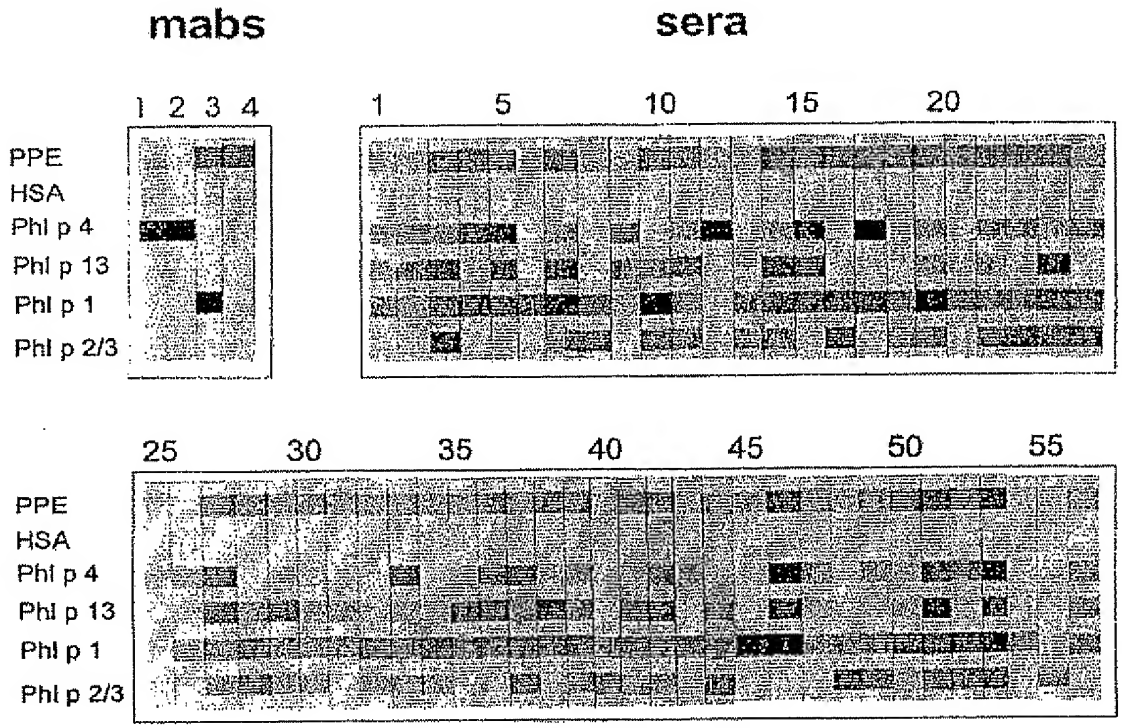


**Fig. 2.** Detection of allergen-specific IgE from patients allergic to grass pollens. Strips of a nitrocellulose membrane carrying timothy grass pollen extract (PPE), human serum albumin (HSA), Phl p 4, Phl p 13, Phl p 1 and group Phl p 2/3 were either incubated with mabs (1,2 = specific for group 4; 3 = specific for group 1; 4 = specific for group 5) or sera (1–56).

between 0.5 and 17.5 kU/l, which represents the upper limit. Regarding Phl p 13, in some cases the staining of the allergen was very feint or absent whilst the amount of specific IgE detected by the ELISA system revealed relatively high values, as for sera 52 and 48. If allowance is made for this observation then the percentage of sera recognized as reacting with Phl p 13 would even be higher.

In order to determine if Phl p 4 and Phl p 13 together account for the whole high molecular mass allergen fraction, inhibition experiments were performed. A serum previously investigated by allergen strip analysis (Fig. 2, no. 47) was pre-adsorbed with either Phl p 4 (Fig. 4, lane 2), Phl p 13 (lane 3) or both allergens (lane 4) and applied to blot strips carrying separated timothy grass pollen extract. Compared with the IgE reaction patterns without pre-adsorption (Fig. 4, lane 1) inhibition with Phl p 4 led to disappearance of a strongly reacting band corresponding to the protein detected by the Phl p 4 specific mab 3C4 (Fig. 4, lane 5). The intensity of the other bands detected including Phl p 1 and Phl p 5 remained unaltered. In contrast, inhibition of the sera with Phl p 13 resulted not only in an elimination of slightly diffuse bands in the range between 50 and 60 kDa, but also to a clearance of the background. Surprisingly, pre-adsorption with Phl p 13 also effected a decrease in IgE-reactivity with protein bands with apparent molecular masses between 90 and 110 kDa. As expected, no effect was observed on the reactivity of Phl p 4. Using both allergens for pre-adsorption, complete reaction of allergen bands in the range of 50–60 kDa as well as a 'background smear' produced by smaller bands was eliminated, but detection of allergens between 25 and 35 kDa, corresponding to Phl p 1 and Phl p 5, was unchanged.

It was considered that a possible explanation for the dissimilar activities of Phl p 4 and Phl p 13 could be related to differing susceptibility to proteolysis. In order to investigate this possibility, both allergens were incubated with the protease Glue-C (Fig. 5). As expected, no effect was observed in the case of Phl p 4, which is known to be trypsin resistant [7]. In contrast, Phl p 13 was completely degraded into smaller fragments covering the range from 50 to 10 kDa. Therefore susceptibility to proteolysis was identified as a distinguishing feature between the two allergens.

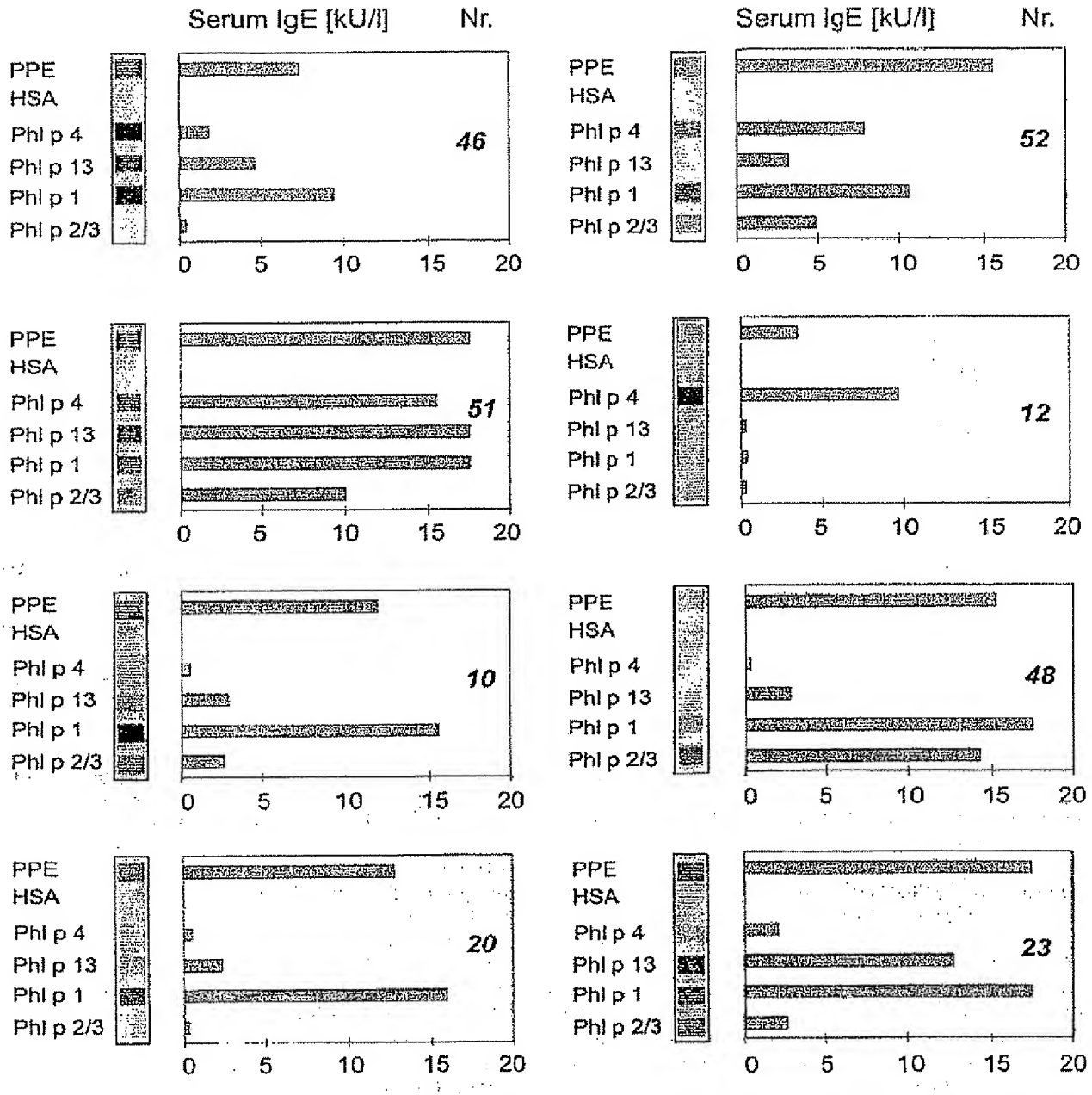


**Fig. 3.** Quantification of allergen-specific IgE. Sera from eight grass pollen allergic patients previously characterized in Fig. 2 were analysed for grass pollen allergens by ELISA measurements. Values below 0.35 KU/L indicate lack of detectable specific IgE. For comparison, allergen strips are displayed on the left.

## Discussion

In previous studies a new high molecular allergen Phl p 13 with an apparent molecular mass of 55–60 kDa has been biochemically purified and the corresponding cDNA (EMBL accession no. AJ238848) expressed in bacteria. As demonstrated by reactivity with monoclonal antibodies, this allergen was different from another high molecular

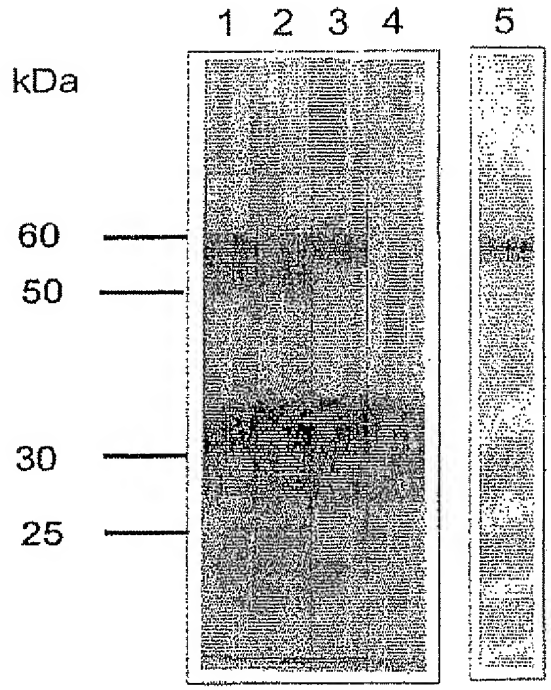


**Fig. 4.** Inhibition of IgE-binding to nitrocellulose-blotted timothy grass pollen extract with purified Phl p 4 and Phl p 13. Strips carrying separated grass extract were incubated with serum no. 47 already characterized in Fig. 2 (lane 1). Lane 2 contains IgE-binding pattern of the serum pre-adsorped with Phl p 4, lane 3 with Phl p 13 and lane 4 with both Phl p 4/Phl p 13. Detection of Phl p 4 was performed with group 4-specific mab 3C4 (lane 5).

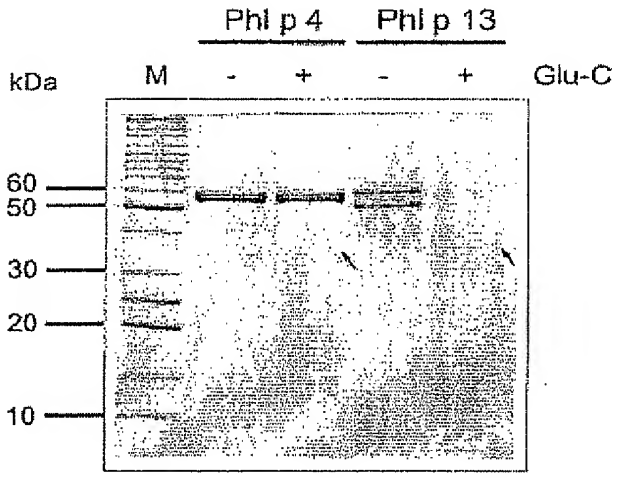


**Fig. 5.** Proteolytic digestion of Phl p 4 and Phl p 13 with Glu-C. Arrows indicate the position of Glu-C [29].

mass allergen designated as Phl p 4. Since both allergens exhibit similar molecular sizes, it has been suggested that former studies evaluating the allergenic importance of Phl p 4 using one dimensional SDS-PAGE indeed detected both allergens (Suck *et al.* submitted). Therefore, investigations were necessary to discriminate between Phl p 4 and Phl p 13 on the polyclonal IgE-level. Since the isoelectric points of the two allergens differ by approximately two units with 7.5 for Phl p 13 and 9.3 for Phl p 4 [8], two-dimensional immunoblotting would represent a reasonable method [22]. However despite possibilities for multiple reprobing of blots [23] only a limited number of sera could have been screened. Consequently, a novel allergen strip method similar to a dot blot was used for this purpose allowing reproducible high throughput IgE-screening with biochemically purified allergens. This analysis revealed that Phl p 4 and Phl p 13 displayed IgE-binding frequencies greater than 50% with 306 sera derived from patients allergic to grass pollens. Hence, both high molecular components have to be judged as major allergens. It seems likely that previous investigations detected not only Phl p 4, but in addition or even exclusively Phl p 13.

Quantification of IgE-binding revealed comparable IgE-binding capacities of Phl p 4 and Phl p 13 and in addition another interesting aspect. Although the IgE-reactivity of some sera was more or less negative for Phl p 13 by allergen strip detection, large amounts of specific IgE directed against this allergen were measured with the ELISA. A possible explanation for this phenomenom might be that different allergen binding mechanisms influence the allergenicity of this molecule. Group 4 ELISA measurements showed that the allergen molecules possess at least two different reactive IgG and IgE epitopes [24,25], whilst IgE-binding was lost after two-dimensional immunoblotting [22]. It has been suggested that Dac g 4 was denatured in such a way that conformational changes occurred [22]. Since Phl p 13 was not harshly denatured for blotting onto nitrocellulose membrane, it seems more likely that it is the manner of the matrix that influences its conformation. Protein binding to nitrocellulose involves hydrophobic interaction [26], which probably effects a unidirectional orientation of some allergens. If this results in masking of epitopes which are recognized by dominant oligoclonal or even monoclonal IgE-pools of some sera, detection will be nearly impossible. In contrast, cyanogen bromide-activated paper, which was used for ELISA measurements, covalently binds proteins in a more random manner. This binding mechanism ensures for the availability of the whole epitope spectrum. Consequently, the number of sera possessing specific IgE against Phl p 13 should be even higher than 50,3% if screening were to be conducted throughout using the ELISA system.

Inhibition experiments were performed to investigate whether Phl p 4 and Phl p 13 represent the whole high molecular mass allergen fraction. Since pre-incubation of serum with both allergens completely eliminated IgE-reactivity in the range of 50–60 kDa, it is likely that this fraction is composed of only two allergens. Differences in the immunostaining intensity between Phl p 4 and Phl p 13 might be due to different proteolytic sensitivity. Group 4 allergens are known to be resistent against proteolytic attack [7], similar to Phl p 1 [26,27]. Thus, intact molecules that were focused sharply after SDS-PAGE were available for IgE-binding. In contrast, Phl p 13 was degraded rapidly with Glu-C and Lys-C (not shown). Assuming proteolytic activity in timothy grass pollen extract, for example group 1 allergens are thought to possess a cysteine proteinase function [28], Phl p 13 is a potential target for degradation. If this is the case it would explain why pre-incubation of sera with Phl p 13 resulted not only in the inhibition of the double band, but also in a decrease of background staining, assuming the smaller bands generating this background reaction are derived from Phl p 13. If this hypothesis is correct then precautions are necessary to protect Phl p 13 during and after extraction of pollens against proteolysis. Preincubation of sera with Phl p 13 also inhibited IgE-binding to very high molecular mass bands. Since Phl p 13 seems to be highly glycosylated (Suck *et al.* submitted), this effect might be due to IgE cross-reactivity between carbohydrate determinants [30,31], assuming glycosylation of these bands.

Another aspect to consider is the possible different extents of glycosylation of Phl p 4 and Phl p 13. Since many glycoproteins behave anomalously when SDS and thiol reagent are in excess [26], it is probable that the highly glycosylated Phl p 13 was not as sharply focused as Phl p 4 after SDS-PAGE. In this case, broadening of the bands would result in less intensive staining compared with Phl p 4, though equal amounts of allergen specific IgE and IgE-binding capacity were determined.

In summary, the data presented in this study clearly demonstrate that both Phl p 4 and Phl p 13 belong to the major allergens of *Phleum pratense*. Since separation of Phl p 4 and Phl p 13 by one dimensional SDS-PAGE is difficult to achieve and Phl p 13 is weakly immunostained, it becomes clear why the important new allergen Phl p 13 has not been recognized until now.

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**PRECEDENTIAL OPINION**

Pursuant to the Board of Patent Appeals and Interference's Standard Operating Procedure 2, the opinion below has been designated a precedential opinion.

UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

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*Ex parte* MAREK Z. KUBIN and RAYMOND G. GOODWIN

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Appeal 2007-0819
Application 09/667,859
Technology Center 1600

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Decided: May 31, 2007

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Before MICHAEL R. FLEMING, *Chief Administrative Patent Judge,* TEDDY S. GRON, TONI R. SCHEINER, ERIC GRIMES, and NANCY J. LINCK, *Administrative Patent Judges.*

LINCK, *Administrative Patent Judge.*

DECISION ON APPEAL

This is a 35 U.S.C. § 134 appeal in the above-referenced case.[1]

We have jurisdiction under 35 U.S.C. § 6(b). We affirm.

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[1] The application was filed September 20, 2000. The real party in interest is Immunex Corporation, a wholly owned subsidiary of Amgen Inc.

## STATEMENT OF THE CASE

The field of the invention is polynucleotides encoding NK (natural killer) Cell Activation Inducing Ligand ("NAIL") polypeptides. (Specification ("Spec.") 1.) NK cells play a role in the "early, innate immune system" and "appear to be closely related to T cells." (*Id.*) Like T cells, the immune response of NK cells "involves direct cytotoxicity and production of various cytokines" that stimulate the immune system. (*Id.* at 3.)

"NK cells have been implicated as mediators of host defenses against infection in humans with varicella zoster, herpes simplex, cytomegalovirus, Epstein-Barr virus, hepatitis B, and hepatitis C viruses." (*Id.* at 3.) They also are "involved in both resistance to and control of cancer spread," including leukemia (*id.* at 3) and "play a . . . role in bone marrow transplant rejection, as well as solid organ transplant rejection." (*Id.* at 4.) Thus, "depletion of NK cells can result in a decreased resistance to target tissue infection by viruses." (*Id.* at 2.) Finally, "a number of human lymphoproliferative disorders of NK cells are known." (*Id.*) "With the function of NK cells so important in this variety of physiological responses, there is a need in the art for methods of controlling NK function." (*Id.*)

NAIL is a cell surface marker, or receptor, on the surface of NK cells that modulates the activity of NK cells. (*See id.* at 2.) Thus, modulation of NAIL activity would be expected to modulate NK cell function, thereby stimulating or inhibiting the immune response.

"CD48 is a membrane glycoprotein found on cells of hematopoietic origin." (*Id.* at 6.) "cDNA clones for CD48 have been isolated" and the

"nucleotide and amino acid sequences of CD48 are known." (*Id.*) Antibodies to CD48 appear to suppress cell mediated immunity. (*Id.* at 6-7.) "The identification of CD48 as a NAIL counter-structure . . . allows the generation of molecules that can modulate the activation of NK . . . cells." (*Id.* at 45.) Thus, the determination of binding to CD48 potentially provides a useful tool to identify active variants of NAIL. (*See, e.g.*, claim 73.)

The claimed subject matter is reflected in representative claim 73:[2]

73. An isolated nucleic acid molecule comprising a polynucleotide encoding a polypeptide at least 80% identical to amino acids 22-221 of SEQ ID NO:2, wherein the polypeptide binds CD48.

The Examiner has rejected claims 73-78 and 80-89 under 35 U.S.C. § 103(a) over the combined teachings of Valiante et al., U.S. Patent No. 5,688,690 (issued Nov. 18, 1997) ("Valiante"); Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, 2.43-2.84 (Cold Spring Harbor, N.Y. 1989) ("Sambrook");[3] and Porunelloor Mathew et al., *Cloning and Characterization of the 2B4 Gene Encoding a Molecule Associated with Non-MHC-Restricted Killing Mediated by Activated Natural Killer Cells and T Cells*, 151 J. IMMUNOL., 5328-5337 (1993) ("Mathew").[4]

The Examiner also has rejected claims 73, 74, 80, and 84-89 under 35 U.S.C. § 112, ¶ 1, for lack of enablement and written description.

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[2] Appellants do not separately argue the claims. Thus, we address each issue with reference to claim 73.

[3] We note Sambrook is incorporated by reference in Valiante (col. 7, ll. 55-57).

[4] This reference is referred to as "Porunelloor" by the Examiner and Appellants.

OBVIOUSNESS UNDER § 103(a)

*The § 103(a) Issue*

The Examiner contends the skilled artisan would have been motivated to isolate the nucleic acid sequence corresponding to NAIL, based on Valiante's disclosure of p38 (which is the same protein as NAIL) and Valiante's express teachings how to isolate p38 cDNA by using conventional techniques, such as taught in Sambrook, including using mAb C1.7, a probe specific for p38. (Answer 11-16.)

Appellants contend: "As in *Deuel*, it is not proper for the Office to use the p38 protein identified in the '690 patent [Valiante] together with the methods such as those described in Sambrook et al. to reject claims drawn to specific sequences." (Br. 19 (citing *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995).)

We frame the § 103(a) issue: Would Appellants' claimed nucleotide sequence have been obvious to one of ordinary skill in the art, based on Valiante's disclosure of p38 and his express teachings how to isolate its cDNA by conventional techniques?

*Findings of Fact Relating to Obviousness*

1. Valiante's p38 protein is a 38kd molecule recognized by mAb C1.7, and is the same protein as Appellants' NAIL, "formerly known as C1.7." (Spec. 10: 29-30. *See also* Answer 14; Spec. 11: 4.)

2. Valiante expressly teaches through a prophetic example how to "isolat[e] the cDNA clone by using [mAb] C1.7, screening the protein expression in the cell transfected with the cDNA library and cloning a corresponding cDNA into a plasmid for sequencing." (Answer 12 (citing Valiante, col. 7, l. 48 through col. 8, l. 7 & example 12, cols. 18-19).)

3. Valiante does not disclose the sequence of p38 recognized by mAb C1.7 or the DNA encoding p38. (*See* Valiante passim; Answer 12.)

4. The DNA and protein sequences of p38, and thus NAIL, could have been obtained by conventional methodologies, such as those taught by Sambrook. (Valiante, col. 7, l. 48 to col. 8, l. 7; *see also* Answer 12.)

5. Sambrook is incorporated by reference in Valiante. (Col. 7, ll. 55-57.)

6. Mathews' cell surface signaling molecule, 2B4, is the mouse version of Valiante's p38, the human version. (Answer 15.)

7. Mathews cloned the gene encoding 2B4 and determined its nucleotide sequence. (Mathews at 5328 (Abstract).)

8. The relevant teachings in Mathews are cumulative to the teachings in Valiante and Sambrook and merely are exemplary of how routine skill in the art can be utilized to clone and sequence the cDNA of a similar polypeptide. (*See* Answer 15.)

9. Appellants employed conventional methods, "such as those outlined in Sambrook," to isolate a cDNA encoding NAIL and determine the cDNA's full nucleotide sequence (SEQ NOS: 1 & 3). (Spec. 10: 29 to 13: 7; Spec. 16: 40 to 17: 1; Spec. 65 (Example 1).)

10. Appellants' claimed polynucleotide is "isolated from [a] cDNA library . . . using the commercial monoclonal antibody C1.7 . . . disclosed by Valiante." (Answer 13. *See also* Spec. 65: 17-32.)

11. As acknowledged by Appellants, "the level of skill in the art is high." (Br. 11 (citing *In re Wands*, 858 F.2d 731, 740, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).)

12. The state of the art had unquestionably advanced significantly during the ten year period between the time the *Deuel* application was filed in 1990 and Appellants' application was filed in 2000. *See In re Wallach*, 378 F.3d 1330, 1333, 71 USPQ2d 1939, 1942 (Fed. Cir. 2004).

13. As acknowledged by Appellants, "methods of making the claimed nucleic acid sequences . . . are known." (Br. 11 (citing *Wands*, 858 F.2d at 740). *See also* Br. 3 ("isolation of clones is well known in the art").)

14. Valiante's disclosure of the polypeptide p38, and a detailed method of isolating its DNA, including disclosure of a specific probe to do so, i.e., mAb C1.7, established Valiante's possession of p38's amino acid sequence and provided a reasonable expectation of success in obtaining a polynucleotide encoding p38, a polynucleotide within the scope of Appellants' claim 73. (*See* Valiante, col. 7, l. 48 to col. 8, l. 7.)

15. As recently clarified by the Federal Circuit, possession of the cDNA encoding NAIL also provided possession of its nucleic acid sequence, i.e., "its identity." *In re Crish*, 393 F.3d 1253, 1258, 73 USPQ2d 1364, 1368 (Fed. Cir. 2004).

16. One of ordinary skill in the art would have had a reasonable likelihood of success that he or she would have been able to obtain the nucleotide encoding NAIL using conventional methods, such as disclosed in Valiante. (*See* col. 7, l. 48 to col. 8, l. 33.)

17. NAIL is "a signal transduction surface molecule (p38) expressed by virtually all human NK cells" and thus plays a role in the immune response. (Valiante, col. 2, l. 65 to col. 3, l. 40.)

18. Thus, one of ordinary skill in the art would have recognized the value of isolating NAIL cDNA, and would have been motivated to apply

conventional methodologies, such as those disclosed in Sambrook and utilized in Valiante, to do so. (*See, e.g.,* Valiante, col. 7, l. 48 to col. 8, l. 33.) *See Alza Corp. v. Mylan Labs*, 464 F.3d 1286, 1289, 80 USPQ2d 1001, 1003 (Fed. Cir. 2006) ("'The presence or absence of a motivation to combine references in an obviousness determination is a pure question of fact.' *In re Gartside*, 203 F.3d 1305, 1316, 53 USPQ2d 1769, 1776 (Fed. Cir. 2000).").

*Discussion of the § 103(a) Issue*

Based on our findings and those of the Examiner, at least one of Appellants' claimed polynucleotides would have been obvious to one of ordinary skill in the art at the time Appellants' invention was made. Regardless of some factual similarities between *Deuel* and this case, *Deuel* is not controlling and thus does not stand in the way of our conclusion, given the increased level of skill in the art and the factual differences. *See In re Wallach*, 378 F.3d 1330, 1334, 71 USPQ2d 1939, 1942 (Fed. Cir. 2004) ("state of the art has developed [since] *In re Deuel*").

Appellants argue the "cited references do not provide an adequate written description of the claimed nucleic acid sequences." (Reply Br. 18 (citing *Noelle v. Lederman*, 355 F.3d 1343, 69 USPQ2d 1508 (Fed. Cir. 2004)). In so arguing, Appellants overlook the distinction between obviousness under § 103 and lack of written description under § 112, § 1. A single, obvious species within a claimed genus renders the claimed genus unpatentable under § 103. Thus, an obvious method of obtaining a single nucleic acid molecule encoding NAIL may be all that is required to show that the presently claimed genus of nucleic acid molecules is unpatentable under § 103. In contrast, as discussed *infra* (see pp. 15-17), the description

of a single species within a claimed genus may not be sufficient to support the patentability of the genus under § 112, ¶ 1. *See University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1567, 43 USPQ2d 1398, 1405 (Fed. Cir. 1997) (noting the court earlier held "a description which renders obvious a claimed invention is not sufficient to satisfy the written description requirement of that invention" and in this case holding disclosure of a species did not provide adequate written description of a genus). *Cf. Eli Lilly & Co. v. Barr Labs*, 251 F.2d 955, 971, 58 USPQ2d 1869, 1880 (Fed. Cir. 2001) ("later genus claim limitation is anticipated by, and therefore not patentably distinct from, an earlier species claim").

With respect to the written description requirement, while "examples explicitly covering the full scope of the claim language" typically will not be required, a sufficient number of representative species must be included "to demonstrate that the patentee possessed the full scope of the [claimed] invention." *Lizardtech, Inc. v. Earth Resource Mapping, Inc.*, 424 F.3d 1336, 1345, 76 USPQ2d 1724, 1732 (Fed. Cir. 2005). Thus, Appellants' argument based on alleged lack of written description in the cited prior art is unavailing.

Appellants heavily rely on *Deuel*. (*See, e.g.*, Br. 19.) To the extent *Deuel* is considered relevant to this case, we note the Supreme Court recently cast doubt on the viability of *Deuel* to the extent the Federal Circuit rejected an "obvious to try" test. *See KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, ___, 82 USPQ2d 1385, 1394, 1396 (2007) (citing *Deuel*, 51 F.3d at 1559). Under *KSR*, it's now apparent "obvious to try" may be an appropriate test in more situations than we previously contemplated.

When there is motivation

> to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under § 103.

*KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, ___, 82 USPQ2d 1385, 1397 (2007). This reasoning is applicable here. The "problem" facing those in the art was to isolate NAIL cDNA, and there were a limited number of methodologies available to do so. The skilled artisan would have had reason to try these methodologies with the reasonable expectation that at least one would be successful. Thus, isolating NAIL cDNA was "the product not of innovation but of ordinary skill and common sense," leading us to conclude NAIL cDNA is not patentable as it would have been obvious to isolate it.

Appellants also argue lack of motivation to combine the cited references. (Br. 20-22; Reply Br. 19-21.) Motivation to combine references "may be found in implicit factors, such as 'knowledge of one of ordinary skill in the art, and [what] the nature of the problem to be solved as a whole would have suggested to those of ordinary skill in the art'." *Alza Corp. v. Mylan Labs.*, 464 F.3d 1286, 1291, 80 USPQ2d 1001, 1004 (Fed. Cir. 2006) (quoting *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1337 (Fed. Cir. 2006)). *See also KSR*, 127 S. Ct. at ___, 82 USPQ2d at 1396 (citing with approval *In re Kahn*, 441 F.3d at 988, 78 USPQ2d at 1336).

More specifically, Appellants argue Mathews "teaches that a human homolog is not expressed," and thus "a person of skill in the art would not be motivated to combine" Mathews with Valiante. (Reply Br. 21.)

Appellants support this argument by quoting from Mathews: "Genomic Southern blots identified a human homologue of the 2B4 gene. However, RNA blot analysis of total RNA isolated from human NK cells suggests that 2B4 gene is not expressed in humans." (Mathews, at 5333, col. 1.)

Rather than teaching away from the combination, as Appellants argue, this language merely indicates conflicting data existed regarding a 2B4 homolog in humans, with some data pointing to the existence of a human homolog. (*See id.*) The quoted language would not have deterred the skilled artisan from obtaining the cDNA corresponding to Valiante's p38, as taught by Valiante, i.e., "from following the path set out in the reference." *In re Gurley*, 27 F.3d 551, 553, 31 USPQ2d 1130, 1131 (Fed. Cir. 1994), *quoted with approval in In re Kahn*, 441 F.3d 977, 990, 78 USPQ2d 1329, 1338 (Fed. Cir. 2006). Moreover, Appellants miscomprehend the value of Mathews. Mathews exemplifies how the cDNA encoding 2B4, the mouse version of Valiante's p38 expressed on all NK cells, can be isolated and sequenced. (*See* Mathews at 5328 (Abstract).) Thus, the teachings of Mathews, when considered as a whole, support the Examiner's § 103 ground of rejection.

PATENTABILITY UNDER § 112, ¶ 1

*The Enablement Issue*

The Examiner found lack of enablement due to the "at least 80% identity language," in the absence of any working examples, other than SEQ ID NOS:1 and 2. He cites examples in the literature in which very small changes in sequence, even one amino acid, yield a different function. (Answer 3-6.)

Appellants respond: "The Office's reasoning ignores the many references that positively demonstrate that proteins can be mutated and maintain a biological function." (Reply Br. 4 (citing numerous publications in support).) Moreover, "the specification provides extensive guidance for creating and screening mutants" (Reply Br. 5) in that it "teaches in detail how to: 1) make variants of SEQ ID NOs: 1 and 2; 2) calculate the percent identity between SEQ ID NOs: 1 and 2 and the variant sequence; and 3) test the variant sequence to determine if it binds to CD48" (Br. 11; Reply Br. 6). Thus, according to Appellants, only routine experimentation would be required to practice the claimed invention. (Reply Br. 9.)

In view of these conflicting positions, we frame the enablement issue as follows: Considering the relevant *Wands* factors, including the prior art teachings cited by the Examiner and Appellants to establish the level of predictability in the relevant art, would undue experimentation have been required to practice the full scope of claim 73?

*The Written Description Issue*

The Examiner bases his written description rejection on the same claim language as the enablement rejection, i.e., "at least 80% identity," and finds Appellants' disclosed sequences inadequate to show "possession of" their claimed genus. (Answer 9 (citing *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997).)

In response, Appellants contend (1) *Lilly* can be distinguished on its facts and (2) the Examiner's position is inconsistent with Example 14 in the

Office's "Synopsis of Application of Written Description Guidelines"[5] (hereafter "*Synopsis*") (www.uspto.gov/web/patents/guides.htm), an example which contains "analysis of [a] claim that is highly similar to the claims at issue." (Reply Br. 13.)

In view of the above, we frame the written description issue: Does Appellants' Specification contain a written description sufficient to show they had possession of the full scope of their claimed invention at the time the application was filed, as required by Federal Circuit precedent?

*Findings of Fact Relating to § 112, ¶ 1*

19. Claim 73 is limited to isolated polynucleotides encoding polypeptides (1) which are "at least 80% identical to amino acids 22-221 of SEQ ID NO:2" (the amino acid sequence for the extracellular domain of NAIL without the signal sequence), and (2) which bind CD48. (*See* claim 73; Spec. 13: 9-18.)

20. The Specification provides two working examples within the scope of claim 73, i.e., a DNA encoding NAIL (SEQ ID NO: 1) and NAIL's coding sequence with accompanying upstream and downstream noncoding sequences (SEQ ID NO: 3). SEQ ID NOs: 1 and 3 both encode the amino acid sequence of SEQ ID NO: 2. (Spec. 10: 29 to 13: 7; Spec. 16: 40 to 17: 1; Spec. 65 (Example 1).)

21. The Specification also discloses the amino acid sequences for three fusion proteins (SEQ ID NOs: 6, 7, & 8) whose nucleotide sequences would fall within the scope of claim 73. (Spec. 25: 30 to 33: 9.)

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[5] Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement, 66 Fed. Reg. 1099 (Jan.5, 2001) ("*Written Description Guidelines*").

22. The Specification does not disclose any variants in which the nucleotide sequence encoding amino acids 22-221 of SEQ ID NO:2 is varied. (Spec. *passim.*)

23. Thus, the Specification does not disclose "which 20% . . . of amino acid residues should be changed in order to maintain the biological functions for binding to CD48." (Answer 5.)

24. The Specification "teaches in detail how to: 1) make variants of SEQ ID NOs: 1 and 2; 2) calculate the percent identity between SEQ ID NOs: 1 and 2 and the variant sequence; and 3) test the variant sequence to determine if it binds to CD48." (Br. 11; Reply Br. 6.)

25. The Specification does not disclose a correlation between function (binding to CD48) and structure responsible for binding to CD48 (other than the entire extracellular domain) such that the skilled artisan would have known what modifications could be made of the very large number of modifications potentially encompassed by claim 73 without losing function. (*See* Spec. *passim*; Answer 10.)

26. At the time Appellants' application was filed, molecular biology was generally an unpredictable art, as evidenced by the references cited by the Examiner. (Answer 4 (citing Robin E. Callard & Andy J.H. Gearing, The Cytokine FactsBook 188-89 (Academic Press 1994); Struyf et al., *Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor*, 28 Eur. J. Immunol. 1262-71 (1998); & Proudfoot et al., U.S. Patent 6,159,711 (Dec. 12, 2000).)

27. At the time Appellants' application was filed, the level of skill in the relevant art (molecular biology) was high, as acknowledged by Appellants. (Br. 11.)

28. "[M]ethods of making the claimed nucleic acid sequences and screening for activity [were] known in the art and described in the specification." (Br. 11-12.)

29. The "experimentation involved to produce other sequences within the scope of the claims" and thus to practice the full scope of claim 73, would have been "well within the skill of those in the art" (Br. 12) and thus would have been routine.

30. One of ordinary skill in the art would not have been required to perform undue experimentation to practice the invention of claim 73.

*Discussion of the Enablement Issue*

In making the above findings, we have considered the relevant *Wands* factors in light of the prior art teachings relied upon by the Examiner and Appellants, and the relevant caselaw. We agree with the Examiner that molecular biology is generally an unpredictable art (and thus was so at the time the application was filed). However, with respect to enablement, the other *Wands* factors weigh in Appellants' favor, particularly "the state of the art" and "the relative skill of those in the art," *In re Wands*, 858 F.2d 731, 736, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), as evidenced by the prior art teachings and Appellants' Specification.

The amount of experimentation to practice the full scope of the claimed invention might have been extensive, but it would have been routine. The techniques necessary to do so were well known to those skilled in the art. *See, e.g., Johns Hopkins Univ. v. Cellpro, Inc.*, 152 F.3d 1342,

1360, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998) ("test [for undue experimentation] is not merely quantitative . . . if it is merely routine"). A "patent need not teach, and preferably omits, what is well known in the art." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). Thus, we conclude the Specification would have enabled the full scope of claim 73.

*Discussion of the Written Description Issue*

In spite of concluding claim 73 would have been enabled, Federal Circuit caselaw compels us to find the written description requirement is not met. *See generally, e.g., University of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 69 USPQ2d 1886 (Fed. Cir. 2004); *Enzo Biochem. Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 63 USPQ2d 1609 (Fed. Cir. 2002); *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997); *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993).

"Although there is often significant overlap" between the enablement and written description requirements, "they are nonetheless independent of each other." *University of Rochester*, 358 F.3d at 921, 69 USPQ2d at 1891. An "invention may be enabled even though it has not been described." *Id.* Such is the situation here. While we conclude one skilled in the art would have been able to make and use the full scope of claim 73 through routine experimentation, we find Appellants did not describe the invention of claim 73 sufficiently to show they had possession of the claimed genus of nucleic acids. *See, e.g., Noelle v. Lederman*, 355 F.3d 1343, 1348, 69 USPQ2d 1508, 1513 (Fed. Cir. 2004) ("invention is, for purposes of the 'written description' inquiry, *whatever is now claimed*").

Claim 73 is to a genus of polynucleotides encoding polypeptides "at least 80% identical to amino acids 22-221 of SEQ ID NO:2" which bind to CD48. Sufficient description to show possession of such a genus "may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Possession may not be shown by merely describing how to obtain possession of members of the claimed genus or how to identify their common structural features. *See University of Rochester*, 358 F.3d at 927, 69 USPQ2d at 1895.

In this case, Appellants have sequenced two nucleic acids falling within the scope of claim 73 and three fusion proteins whose nucleotide sequences would fall within the scope of claim 73. None of these sequences varies amino acids 22-221 of NAIL, and thus these sequences are not representative of the genus.

Appellants also have described how to make and test other sequences within claim 73 sufficiently to satisfy the enablement requirement. However, they have not described what domains of those sequences are correlated with the required binding to CD48, and thus have not described which of NAIL's amino acids can be varied and still maintain binding. Thus, under *Lilly* and its progeny, their Specification would not have shown possession of a sufficient number of sequences falling within their potentially large genus to establish possession of their claimed genus. *Cf. Enzo*, 323 F.3d at 964, 63 USPQ2d at 1612 ("if the functional characteristic of . . . binding to [CD48] were coupled with a disclosed correlation between

that function and a structure that is sufficiently known or disclosed," the written description requirement may be met).

Without a correlation between structure and function, the claim does little more than define the claimed invention by function. That is not sufficient to satisfy the written description requirement. *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("definition by function ... does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is").

With respect to Appellants' reliance on hypothetical Example 14 in the Office's *Synopsis*, "[c]ompliance with the written description requirement is essentially a fact-based inquiry that will 'necessarily vary depending on the nature of the invention claimed.'" *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991) (quoting *In re DiLeone*, 436 F.2d 1404, 1405, 168 USPQ 592, 593 (CCPA 1971)), *quoted with approval in Enzo*, 323 F.3d at 963, 63 USPQ2d at 1612. While the *Written Description Guidelines* and the hypothetical examples in the Office's *Synopsis* can be helpful in understanding how to apply the relevant law (as it existed in 2001 when the Guidelines were adopted), they do not create a rigid test.

Based on the above, we find the written description requirement of § 112, ¶ 1, is not met.

## CONCLUSION

In summary, with respect to claim 73, we affirm the § 103(a) rejection, reverse the § 112, ¶ 1, enablement rejection, and affirm the § 112, ¶ 1, written description rejection.

Pursuant to § 41.37(c)(1)(vii)(2006), we also affirm the rejection of claims 74-78 and 80-89 under § 103(a); reverse the § 112, ¶ 1, enablement rejection of claims 74, 80, and 84-89; and affirm the § 112, ¶ 1, written description rejection of claims 74, 80, and 84-89, as these claims were not argued separately.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a)(1)(iv)(2006).

AFFIRMED

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